AMRL-TR-78-86

ENVIRONMENTAL QUALITY RESEARCH, USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMINANTS

Third Annual Report

JAN SCHERFIG
PETER S. DIXON
CAROL A. JUSTICE

THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
UNIVERSITY OF CALIFORNIA, IRVINE
IRVINE, ORANGE COUNTY, CALIFORNIA 92664

NOVEMBER 1978

Approved for public release; distribution unlimited.

AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

NOTICES

When US Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from Aerospace Medical Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with Defense Documentation Center should direct requests for copies of this report to:

Defense Documentation Center
Cameron Station
Alexandria, Virginia 22314

TECHNICAL REVIEW AND APPROVAL

AMRL-TR-78-86

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

A. A. Thomas

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

AIR FORCE/56780/11 January 1979 — 100

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: AMRL-TR-78-86	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE *(and Subtitle)*: Use of Unicellular Algae for Evaluation of Potential Aquatic Contaminants Third Annual Report	5. TYPE OF REPORT & PERIOD COVERED: Annual Report 1 June 1977 - 31 May 1978
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Jan Scherfig, Peter S. Dixon, and Carol A. Justice	8. CONTRACT OR GRANT NUMBER(s): F-33615-76-C-5005
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Regents of the University of California, University of California, Irvine, California 92717	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 62202F 6302/04/17
11. CONTROLLING OFFICE NAME AND ADDRESS: Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, OH 45433	12. REPORT DATE: November 1978
	13. NUMBER OF PAGES: 32
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)*: Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Propellants	Algal Bioassays	Dunaliella tertiolecta
Rocket Fuels	Safe Concentrations	Selenastrum capricorntum
Hydrazine	Effective Concentrations	monomethylhydrazine
Unsymmetrical	Dimethylhydrazine	

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Selenastrum capricorntum and Dunaliella tertiolecta were used as test algae in bioassays to determine the toxic and/or biostimulating effects of hydrazine and methylated hydrazines in various freshwater and marine aquatic environments. Standard batch algal assay procedures were used to determine safe concentrations and effective concentrations for the compounds studied. Chemical analyses were performed to determine compound stability under the various test conditions.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

PREFACE

This is the Third Annual Report of work performed under the Air Force Contract AF33615-76-C-5005 and covers the period June 1, 1977 to May 31, 1978. The project is entitled "Use of Unicellular Algae for Evaluation of Potential Aquatic Contaminants." Research was conducted by the Water Resources Laboratory, School of Engineering, University of California, Irvine. The investigation was designed to expand the knowledge of toxic and biostimulatory responses of unicellular algae to hydrazine propellants and to aid Air Force personnel in assessing the environmental impact of compounds which may be released into the aquatic environment.

Contract monitors were Lt/ Colonel Roger C. Inman and Major C. B. Harrah who assumed responsibility later in the year as Chief, Environmental Quality Branch of Toxic Hazards Division, AMRL, Wright Patterson Air Force Base, Dayton, Ohio. Principal investigators were Jan Scherfig, Civil and Environmental Engineering and Peter S. Dixon, Department of Ecology and Evolutionary Biology, University of California, Irvine. Project coordinator was Mrs. Carol Justice.

The authors gratefully acknowledge the assistance of Miss Mahin Talebi for her efforts in the overall conduct of this study.

TABLE OF CONTENTS

LIST OF TABLES

SUMMARY

This report describes the determination of the relative safety of hydrazine, unsymmetrical dimethylhydrazine, and monomethylhydrazine if they are released into the aquatic environment, for example, by accidental spills. The results will be used by the U.S. Air Force to determine how activities which involve these compounds can be conducted in conformance with the National Environmental Policy Act.

This report provides detailed results from the work conducted during 1977/78 and presents a comprehensive summary of all the testing and results of hydrazine toxicities obtained in the previous two years of investigation.

The results of this year's work are summarized in Table 1. This indicates the initial concentrations of compound in µℓ/ℓ which result in no effect (Safe Concentration) and in a 50 percent growth reduction on Day 6 (EC_{50}) on the basis of total cell volume.

TABLE 1

SUMMARY OF TOXICITY OF HYDRAZINE COMPOUNDS TO ALGAE

Type of Water Test Organism	Compound					
	Hydrazine		MMH		UDMH	
	SC	EC_{50}	SC	EC_{50}	SC	EC_{50}
Oligotrophic Freshwater *S. capricornutum*	0.001	0.03	0.2	0.5	2.0	5.0
Oligotrophic Seawater *D. tertiolecta*	0.0005	0.0008	0.8	1.1	0.1	2.3

Toxicity of the compounds studied was directly correlated with compound stability during the bioassays. Hydrazine, the most stable in the various algal growth media, was the most toxic, while UDMH, the least stable, was also the least toxic.

INTRODUCTION

Increasing emphasis on the preservation of environmental quality has made it necessary to determine the effects of substances released into the environment. Hydrazine propellants are used as rocket fuels for current space launch vehicles and are proposed for use in future space systems. Their use presents the possibility of spillage into the aquatic environment and the effects of these compounds in various types of aquatic systems must therefore be determined.

Algal bioassays provide a firm basis for assessing the impact of possible aquatic contaminants on algae over a wide range of nutrient and salinity levels. Algae are particularly significant as major primary producers in all aquatic food chains.

OBJECTIVES

Research objectives for the past year have been directed towards the determination of toxic and/or biostimulatory effects of three hydrazine compounds to several species of unicellular green algae under different natural water conditions. Compounds studied include hydrazine, unsymmetrical dimethylhydrazine (UDMH), and monomethylhydrazine (MMH).

Quantitative bioassays were utilized to determine concentrations of the test compound necessary to cause a response of the bioassay organism. Both freshwater and marine bioassays were conducted under varying test conditions to simulate a range of aquatic ecosystems, such as oligotrophic lakes, eutrophic lakes, lakes of intermediate trophic status, estuaries and the open sea. The overall goals have been to provide information about relative safety of the compounds for environmental impact statements and determine threshold limits under which the Air Force can operate within the National Environmental Policy Act.

Specific Objectives

1. Determining the safe concentration (SC) for each of the compounds under the various test conditions. The SC is defined as the highest concentration of test compound that can be administered without causing a detectable difference in maximum standing crop. (Maximum standing crop is considered to have been reached when the increase in algal growth is less than five percent per day.)
2. Determining the median effective concentration (EC_{50}) for the compounds under the various test conditions. The EC_{50} is that concentration of test compound which causes a fifty percent reduction in algal growth when compared to controls with no test compound added.
3. Determining how much the SC and EC_{50} doses would be affected by changes in water quality and differences in algal species.
4. Assessing compound stability during storage and under various environmental conditions.

In addition, during the present year, several subsidiary goals which were involved in answering the above questions became apparent. These included:

5. Standardization of the hydrazine used in this study and related studies conducted in other laboratories.
6. Evaluation of Chlorella stigmatophora as a marine test organism compared with the alga used previously, Dunaliella tertiolecta.
7. Development of axenic batch test procedures.

Workplan

The results obtained in the initial screening experiments with hydrazine were reported in the First Annual Report of this Project (1976), with the more detailed studies constituting the Second Annual Report (1977). In the Second Annual Report, the compounds tested and the media in which they were evaluated are shown in Table 2.

TABLE 2

COMPOUNDS TESTED AND TEST CONDITIONS OF 1976/77 BIOASSAYS

COMPOUND	TEST CONDITIONS	
	Type of Water	Nutrient Level
Hydrazine	Freshwater	10% SAAM nutrients
	Freshwater	33% SAAM nutrients
	Freshwater	100% SAAM nutrients
UDMH	Freshwater	10% SAAM nutrients
	Freshwater	33% SAAM nutrients
	Freshwater	100% SAAM nutrients
	Marine, 35 ppt salinity	33% SAAM nutrients
MMH	Marine, 35 ppt salinity	33% SAAM nutrients

The present report continues this work and a similar summary of compounds tested and the media used are given in Table 3.

These experiments were designed to extend the scope of the work completed in the previous year and simulate the range of conditions likely to be encountered in bays and estuaries which receive drainage from sylvan, agricultural or urban runoff.

TABLE 3

COMPOUNDS TESTED AND TEST CONDITIONS OF 1977/78 BIOASSAYS

COMPOUND	TEST CONDITIONS	
	Type of Water	Nutrient Level
Hydrazine	Marine, 16 ppt salinity	10% SAAM nutrients
	Marine, 16 ppt salinity	33% SAAM nutrients
	Marine, 24 ppt salinity	10% SAAM nutrients
	Marine, 24 ppt salinity	33% SAAM nutrients
UDMH	Marine, 35 ppt salinity	10% SAAM nutrients
	Marine, 24 ppt salinity	10% SAAM nutrients
	Marine, 16 ppt salinity	33% SAAM nutrients
	Marine, 16 ppt salinity	10% SAAM nutrients
	Marine, 16 ppt salinity	33% SAAM nutrients
MMH	Freshwater	10% SAAM nutrients
	Freshwater	33% SAAM nutrients
	Freshwater	100% SAAM nutrients
	Marine, 35 ppt salinity	10% SAAM nutrients
	Marine, 35 ppt salinity	33% SAAM nutrients
	Marine, 24 ppt salinity	10% SAAM nutrients
	Marine, 24 ppt salinity	33% SAAM nutrients
	Marine, 16 ppt salinity	10% SAAM nutrients
	Marine, 16 ppt salinity	33% SAAM nutrients

The nutrient levels for these experiments are equivalent to the following natural conditions:

Freshwater: The ranges used correspond to oligotrophic (10% SAAM nutrients), intermediate (33% SAAM nutrients) and eutrophic (100% SAAM nutrients).

Seawater: The seawater experiments were conducted at a range of salinity and nutrient level. At a salinity of 35 ppt, the lower nutrient level (10% SAAM nutrients) is equivalent to open-sea conditions, while the higher level (33% SAAM nutrients) is equivalent to conditions encountered near to sewage outfalls or off the mouths of estuaries where nutrient-rich drainage from agriculture occurs. The experiments at lower salinities (16 ppt, 24 ppt) and the same two levels of nutrients (10% SAAM, 33% SAAM) simulate conditions found in estuaries of differing nutrient status.

CONCEPTS AND METHODS

The analytical and assay procedures have been reported in detail in the previous annual report. Only modifications and new procedures developed during the present year are discussed in this annual report.

CONCEPTS

Three key concepts are used in this work to form the basis for the conclusions regarding the effects of the different hydrazine compounds in the aquatic environment.

Biological Growth Measures

The main concept used is the measure of biological activity. Several measures can be used including oxygen production rates, specific growth rates, and maximum biomass produced. During the early periods of this investigation extensive work was done to evaluate the applicability and methods to interpret the results obtained with each of these three parameters. Based on that work it was decided that two measures should be used to evaluate the effects of hydrazines.

The first measure is maximum standing crop. The maximum standing crop is defined as the amount of algal growth (as cell numbers or total cell volume) obtained when algal growth had culminated. This is determined as the time when the increase in algal growth has stopped or decreased to less than five percent per day.

One major difficulty has been encountered with this measure of the effect of hydrazine compounds on growth of algae. The difficulty is related to the instability of hydrazine compounds in natural waters. The hydrazine compounds will decompose within a few days compared to the normal 10-15 days required to reach the maximum standing crop. Thus, even though there is a significant short-term toxic effect from hydrazines, after 10 days the net effect is sometimes biostimulatory, perhaps because the nitrogen in the hydrazines becomes available to the algae.

In order to determine the absolute and relative toxicity of the different hydrazine compounds it was therefore decided to determine the effect by the relative growth compared to a control sample after six, eight, and ten days of growth. The relative growth figures are then used to determine the toxic concentration of the hydrazine compounds.

Toxic Concentrations

Two different and complementary measures have been selected to quantify the toxic levels of the hydrazine compounds. The first of these is the Safe Concentration (SC). The Safe Concentration is the maximum concentration of a hydrazine which can be present without causing a statistically detectable difference in algal biomass.

The second measure used is the median effective concentration (EC_{50}) which is that concentration which results in a 50 percent reduction in biomass at a given time when compared to the control.

Statistical Determination of Toxic Concentrations

The toxic concentrations have been determined on the basis of Analyses of Variance and t-tests for the specific experiments combined with interpolation between concentrations of hydrazines added. In addition, the results obtained both this year and last year are currently being refined by means of Probit Analyses and all results will be presented on this basis in the future reports as well as in the final summary report.

METHODS

Algal bioassays were conducted in accordance with Standard Methods (American Public Health Association, 1975) and the Algal Assay Procedure: Bottle Test (United States Environmental Protection Agency, 1971) in order to determine the safe concentration (SC) and median effective concentration (EC_{50}).

Modifications of the Algal Assay Procedure included the following:

1. A larger volume of medium was used (250 ml/500 ml flasks) but has been shown to have no effect with the auxiliary aeration system that was used.
2. Temperature control was 25 + 3°C.
3. All compounds contained in the growth medium were added in a particular order before filtration in order to prevent iron precipitation. The order of additions was sodium bicarbonate, magnesium sulfate, calcium chloride, potassium orthophosphate (mono-H), magnesium chloride, sodium nitrate and trace metals including a chelating agent.

Algal bioassays were conducted in two steps: (1) a broad screening series and (2) a fine evaluation analysis. First, a preliminary series of replicate flasks containing the algal growth medium was dosed with a broad range of concentrations (e.g. from 0.001 to 10 ppm) of the test compound. Flasks were seeded with the appropriate test organism and algal growth (both total cell number and total algal volume) was monitored with an electronic particle counter (Coulter model TA II with population accessory) until at least the control flasks without test compound reached the maximum standing crop. The maximum standing crop or maximum biomass is defined as having been achieved when the biomass increase is 5% or less per day. In this way it was possible to determine the approximate concentration where the SC and EC_{50} would be expected to occur. Then another series of flasks containing growth medium was dosed with this narrow range of concentrations of the test compound. All flasks were seeded to an initial concentration of 1×10^6 cells/ℓ with the appropriate algal species. Selenastrum capricornutum was the test organism for freshwater bioassays and Dunaliella tertiolecta was the marine test organism. Algal growth was monitored as described above and the SC

and EC_{50} concentrations were determined. The Standard Algal Assay Medium (SAAM) was the growth medium for freshwater bioassays and modified Burkholder's artificial seawater (ASW) with varying SAAM levels of nitrogen and phosphorus was the medium for marine algal assays. Compounds tested included hydrazine, unsymmetrical dimethylhydrazine (UDMH) and monomethylhydrazine (MMH).

Chemical Procedures

The analytical procedures to determine hydrazine concentrations have been improved and refined during the year especially with respect to determination of MMH. The results of these improvements are shown in the Appendix.

Test Compound Concentration

Test compounds were freshly prepared by serial dilution from the stock bottle immediately before being added to the bioassay flasks containing the algal cells. Five replicate flasks were prepared separately for each of the desired initial concentrations of test compound. A sample was removed from at least three of the bioassay flasks and analyzed chemically to determine whether the desired and actual concentrations were in agreement. In some cases the limit of detection for a particular compound was higher than the desired initial concentration and direct verification of the amount present was not possible. In most cases, the "desired" and "actual" initial concentrations were in very good agreement.

Hydrazine Compound Stability in Solution

A series of tests were performed to determine the relative stability of the three hydrazine compounds under varying simulated environmental conditions. The results showed a very significant effect of compound stability on the results of the bioassays. Consequently a comprehensive study was made and the results are presented.

RESULTS AND DISCUSSION

The investigation conducted this year has been divided into three major inter-related areas. The first of these is the stability of hydrazine compounds in aquatic environments. The second and most important area is the environmental effects of hydrazines in the aquatic environment including both toxic and biostimulatory responses. The third area of investigation relates to improvements in the available analytical methods and verification of actual concentrations of hydrazines based on GC/MS measurements.

STABILITY OF HYDRAZINE COMPOUNDS

One of the most important factors that relate to determination of the toxic and biostimulatory effects of hydrazines is this rate of decomposition into components which are not toxic to the test organisms studied.

In preliminary investigations we observed that the rate was dependent upon the type of water, the initial concentration of the hydrazine compound, and the amount of trace metals present. Results of decomposition experiments in fresh waters were reported in the previous annual report (AMRL-TR-77-53); the decomposition times were of the same order of magnitude as the time required for completion of an algal assay. The net effect of this combination was in many cases an observed initial growth depression which was subsequently followed by a final growth increase compared with the control assays. Preliminary screening tests indicated that this might be even more significant for the seawater conditions evaluated during this investigation and detailed tests were therefore conducted varying concentrations of SAAM, SAAM macronutrient solution, SAAM trace metal solution and deionized water. These tests of hydrazine stability were carried out in deionized water and ASW medium with a salinity of 24 ppt and a nutrient level of 33% SAAM. Concentrations in ASW and deionized water as a function of time are shown in Table 4 and Table 5 for hydrazine and in Tables 6 and 7 for UDMH. The results of these experiments show that both hydrazine and UDMH are stable under the test conditions in deionized aerated water. Hydrazine is also relatively stable in artificial seawater (ASW) at 24 ppt salinity whereas 80-100 percent of added UDMH will have decomposed within forty-eight hours.

The decomposition results are very important when evaluating the bioassay results presented and they are discussed in connection with the overall evaluation of the relative hydrazine compound toxicity.

ENVIRONMENTAL EFFECTS OF HYDRAZINE COMPOUNDS

The environmental effects of the three hydrazine compounds as determined under different aquatic conditions of salinity and nutrient concentrations have been outlined. The detailed results have been presented in the monthly reports during the last two years. These results are summarized below for each of the compounds tested. The number of algal cells grown as well as the total algal cell volumes have been used as the two measures of potential effects on the test species. The concentrations of hydrazine compounds that have a potential effect have been determined both on the basis of Safe Concentration (SC) and Median Effective Concentration (EC_{50}) as discussed above.

The Second Annual Report gave the results of freshwater experiments conducted in a range of nutrient conditions ranging from low oligotrophic to high eutrophic, together with the results of experiments conducted in artificial seawater (ASW), at 35 ppt salinity, at two levels (10 percent, 33 percent) of SAAM nutrient conditions. The lower level of SAAM nutrient conditions is that salinity equivalent to open-sea coastal waters while the higher level is equivalent to conditions encountered near to sewage outfalls or at the mouths of estuaries where nutrient-rich drainage from agriculture occurs. During the present year, ASW of lower salinities (16 ppt, 24 ppt) were used as the test media, with the same two levels of SAAM nutrient conditions. These experiments were designed to extend the scope of the work done in the previous year and simulate the range of condition likely to be found in estuaries.

Environmental Effects of Hydrazine

The hydrazine concentrations were prepared by successive dilution. As the detection limit for hydrazine (0.005 µℓ/ℓ) in the method used for analysis is greater than most of the initial concentrations used for the experiments, direct verification of the amount present was not possible. Once the experiments had begun, hydrazine could not be detected in any of the flasks after two days. Flasks were seeded with Dunaliella tertiolecta to an initial cell concentration of 1×10^6 cells/ℓ, and algal growth was monitored on days 6, 8, and 10.

Algal bioassays were conducted utilizing five replicates for each of the following initial hydrazine concentrations: 0.0000, 0.0001, 0.0005, 0.0010, 0.0030, 0.0050, and 0.0100 µℓ per liter of ASW medium.

Tables 8 and 9 present a summary of SC and EC_{50}, respectively. Using the results obtained on growth day 6 as a representative indication it can be seen that there is no significant difference between the results obtained using cell number or cell volume as the response parameter. The Safe Concentrations range from 0.001 µℓ/ℓ under oligotrophic freshwater conditions to 0.0001 µℓ/ℓ under oligotrophic brackish conditions. The corresponding EC_{50} range is from 0.03 µℓ/ℓ to 0.0004 µℓ/ℓ.

There is little or no difference between the SC and EC_{50} values obtained at the various brackish and seawater salinity levels. This indicates that the cause of the range is not salinity variation or amount of nutrients present. Instead, the observed differences in SC and EC_{50} values indicate that there is a very significant difference in sensitivity between the two alga used under freshwater and brackish/seawater conditions respectively. The saltwater alga, Dunaliella tertiolecta is affected by concentrations of hydrazine which are one order of magnitude lower than the concentration required to affect the growth of Selenastrum capricornutum.

TABLE 4

STABILITY OF HYDRAZINE IN ASW AT 24 PPT SALINITY WITH 33% SAAM NUTRIENTS

Hydrazine Concentration - µℓ/ℓ			
Initial	22 hrs.	48 hrs.	96 hrs.
0.00	0.00	0.00	0.00
0.02	0.02	0.01	0.01
0.03	0.03	0.02	0.01
0.04	0.03	0.03	0.02
0.05	0.04	0.04	0.03
0.06	0.05	0.05	0.03
0.07	0.06	0.06	0.05
0.08	0.07	0.06	0.05
0.10	0.09	0.08	0.07

TABLE 5

STABILITY OF HYDRAZINE IN DEIONIZED WATER

Hydrazine Concentration - µℓ/ℓ			
Initial	22 hrs.	48 hrs.	96 hrs.
0.00	0.00	0.00	0.00
0.02	0.02	0.02	0.02
0.03	0.03	0.03	0.03
0.04	0.04	0.04	0.04
0.05	0.05	0.05	0.05
0.06	0.07	0.07	0.07
0.07	0.07	0.07	0.07
0.08	0.08	0.08	0.08
0.10	0.10	0.10	0.09

TABLE 6

STABLIITY OF UDMH IN ASW AT 24 PPT SALINITY WITH 33% SAAM NUTRIENTS

UDMH CONCENTRATION - µℓ/ℓ				
Initial		20 hours	44 hours	90 hours
Desired	Analyzed			
0.00	0.0	0.00	0.00	0.00
0.20	0.23	0.19	0.00	0.00
0.40	0.37	0.23	0.00	0.00
0.80	0.71	0.31	0.00	0.00
1.00	0.95	0.35	0.00	0.00
1.50	1.52	0.39	0.23	0.31
2.00	1.92	0.47	0.31	0.35
2.50	2.61	0.55	0.39	0.41
3.00	2.80	0.59	0.45	0.46
4.00	4.14	0.91	0.75	0.65

TABLE 7

STABILITY OF UDMH IN DEIONIZED WATER

UDMH CONCENTRATION - µℓ/ℓ				
Initial		20 hours	44 hours	90 hours
Desired	Analyzed			
0.00	0.00	0.00	0.00	0.00
0.40	0.37	0.34	0.37	0.30
0.80	0.84	0.71	0.74	0.71
1.00	1.03	0.93	0.96	0.89
1.50	1.50	1.44	0.41	0.22
2.00	2.00	1.92	1.96	1.81
3.00	2.99	2.92	2.92	2.79
4.00	3.99	3.89	3.81	3.81

TABLE 8

SAFE CONCENTRATION DOSES FOR HYDRAZINE (as µℓ/ℓ) UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Water Quality		Day 6		Day 8		Day 10	
Salinity	Eutrophication level	Number	Volume	Number	Volume	Number	Volume
Fresh	Oligotrophic	0.00100	0.00100	0.00200	0.00200	0.00200	0.00200
Fresh	Eutrophic	0.00100	0.00100	0.01000	0.01000		
Fresh	Very Eutrophic	0.00500	0.00500				
Brackish 16 ppt	Oligotrophic	0.00005	0.00010	0.00008	0.00050		
Brackish 16 ppt	Eutrophic	<0.00005	<0.00050	0.00050	0.00050	0.00050	0.00050
Brackish 24 ppt	Oligotrophic	0.00010	0.00010	0.00010	0.00010	0.00010	0.00050
Brackish 24 ppt	Eutrophic	0.00010	0.00010	0.00050	0.00010	0.00100	0.00050
Sea Water 35 ppt	Oligotrophic	0.00050	0.00050	0.00050	0.00080	0.00050	0.00080
Sea Water 35 ppt	Eutrophic	0.00100	<0.00010	0.00010	0.00100		

TABLE 9

FIFTY PERCENT EFFECTIVE DOSES FOR HYDRAZINE (as µℓ/ℓ) UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Water Quality		Day 6		Day 8		Day 10	
Salinity	Eutrophication level	Number	Volume	Number	Volume	Number	Volume
Fresh	Oligotrophic	0.00200	0.03000	0.02000	0.03000	0.07000	0.08000
Fresh	Eutrophic	0.01300	0.01300	0.03700	0.03200		
Fresh	Very Eutrophic	0.00600	0.00700				
Brackish 16 ppt	Oligotrophic	0.00040	0.00045	0.00080	0.00085		
Brackish 16 ppt	Eutrophic	0.00070	0.00070	0.00170	0.00130	0.00170	0.00130
Brackish 24 ppt	Oligotrophic	0.00040	0.00040	0.00070	0.00070	0.00090	0.00140
Brackish 24 ppt	Eutrophic	0.00130	0.00160	0.00180	0.00160	0.00190	0.00140
Sea Water 35 ppt	Oligotrophic	0.00080	0.00080	0.00080	0.00100	0.00200	0.00400
Sea Water 35 ppt	Eutrophic	0.00110	0.00140	0.00370	0.00310		

Based on these results additional work is being conducted evaluating a number of different test alga to determine the range of sensitivity that can be expected in natural algal populations.

Environmental Effects of Monomethylhydrazine

The present studies examined the responses of the freshwater alga, Selenastrum capricornutum, and the marine flagellate, Dunaliella tertiolecta, to different ranges of nutrient conditions, and for the latter a range of salinities. In all cases, five controls without MMH and five replicate flasks for each concentration of MMH were seeded to an initial concentration of 1×10^6 cells per liter. Algal growth (both cell number and total cell volume) and MMH concentration were measured at intervals for at least 10 days and in some cases for as long as 31 days.

The bioassays were conducted using initial MMH concentrations of 0.20, 0.40, 0.60, 0.80, 1.00, and 2.00 µℓ of MMH per liter in the freshwater samples and from 0.40 to 2.4 µℓ of MMH per liter in the brackish/seawater tests.

A typical example of the average results for one bioassay test condition is presented in Table 10 together with the corresponding SC and EC_{50} values in Table 11.

TABLE 10

INITIAL MMH CONCENTRATIONS AND PERCENT ALGAL GROWTH IN ASW AT 35 PPT SALINITY WITH 10% SAAM NUTRIENTS

Initial MMH µℓ/ℓ	PERCENT ALGAL GROWTH					
	Day 6		Day 8		Day 10	
	Number	Volume	Number	Volume	Number	Volume
0.0	100	100	100	100	100	100
0.4	103	103	117	115	112	103
0.8	95	94	116	120	102	98
1.2	53	43	106	96	101	102
1.6	2	2	72	57	105	100
2.0	< 1	< 1	58	47	109	98
2.4	< 1	< 1	1	1	1	1

Low concentrations of MMH appear to be somewhat biostimulating and result in greater cell numbers and volumes when compared to the controls with MMH added. However, this increased growth is generally not significant statistically.

TABLE 11

SAFE CONCENTRATIONS AND FIFTY PERCENT EFFECTIVE CONCENTRATIONS FOR MMH (as µℓ/ℓ)AT 35 PPT SALINITY WITH 10% SAAM NUTRIENTS

Growth Day	SC		EC_{50}	
	Number	Volume	Number	Volume
6	0.8	1.2	1.2	1.0
8	1.6	1.6	2.1	1.8
10	2.0	2.0	2.3	2.3

The results of all the MMH assays are summarized in Tables 12 and 13 for SC and EC_{50}, respectively. The results show that the Safe Concentrations based on cell volumes measured at Day 6 range from <0.2 µℓ MMH/ℓ in freshwater to 0.8 µℓ MMH/ℓ in saltwater. The corresponding EC_{50} values range from 0.3 µℓ MMH/ℓ to 1.2 µℓ MMH/ℓ.

Examination of Coulter Counter data for the freshwater assays with Selenastrum capricornutum as the test organism showed a progressive increase in mean algal cell volume as the MMH concentration increased. This increase in cell size at the higher MMH concentration seems to indicate that MMH does not kill the algal cells but inhibits cell division because of interference with some metabolic pathway. Therefore, the cells continue to enlarge but do not divide. The metabolic interference continues to exist for sometime after the MMH concentration drops to a level which, initially, would not be growth inhibiting. Once the metabolic interference is overcome, the cells begin to divide very rapidly so that the algal culture consists of new, slightly smaller cells. Further evidence of the growth inhibiting effect rather than lethality of MMH is the fact that all flasks which had an initial 1.0 µℓ/ℓ concentration grew well after 30 to 40 days.

Environmental Effects of Unsymmetrical Dimethylhydrazine

Algal assays were conducted using a range of UDMH concentrations from 0.0 to 3.0 µℓ/ℓ. The results of these assays are summarized in Tables 14 and 15 for the Safe Concentrations and Fifty Percent Effective Concentrations. Using the values for Day 6, the results show that the Safe Concentration, based on total cell volume as the key parameter, range from 5.0 µℓ/ℓ in eutrophic freshwater to 0.1 µℓ/ℓ in seawater. The Safe Concentrations, determined by evaluating cell numbers, rather than total cell volumes, are lower for freshwater conditions. This indicates that, as with MMH, UDMH inhibits cell division.

Comparison of the Safe Concentrations and Fifty Percent Effective Concentrations for Hydrazine Compounds

The results of all the experiments can be summarized to show the relative toxicity of the three hydrazine compounds under the range of water qualities and organisms tested. The summary results in Table 16 show that hydrazine is the most toxic of the three compounds under both freshwater and seawater assay conditions. The Safe Concentration for hydrazine is several hundred times lower than the Safe Concentration for UDMH measured after six days of growth. Both SC and EC_{50} are lower under seawater assay conditions than under freshwater conditions for hydrazine and UDMH with the reverse for MMH. This difference appears to be due to the different sensitivity of the two test algae used in fresh and sea water.

Results in Table 16 are initial concentrations of compound in µℓ/ℓ which result in no effect (Safe Concentration) and in a 50 percent growth reduction on Day 6 on the basis of total cell volume biomass.

One of the explanations for the apparent higher toxicity (lower Safe Concentrations and Fifty Percent Effective Concentrations) for hydrazine compared with MMH and UDMH is the much greater stability of the former. Thus, in terms of potential impact on the aquatic environment, the use of MMH and UDMH is recommended as being preferable to hydrazine, based on the results obtained in this investigation.

TABLE 12

SAFE CONCENTRATION DOSES FOR MMH (as µℓ/ℓ) UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Water Quality		Day 6		Day 8		Day 10	
Salinity	Eutrophication level	Number	Volume	Number	Volume	Number	Volume
Fresh	Oligotrophic	<0.200	<0.200	<0.200	<0.200	<0.200	<0.200
Fresh	Eutrophic	<0.200	<0.200	<0.200	<0.200	<0.200	<0.200
Fresh	Very Eutrophic	<0.200	<0.200	0.200	0.200	<0.200	<0.200
Brackish 16 ppt	Oligotrophic	0.041	0.024	0.024	0.024		
Brackish 16 ppt	Eutrophic	0.040	0.040	0.040	0.040	0.040	0.080
Brackish 24 ppt	Oligotrophic	0.800	0.800	0.800	0.800	1.600	0.800
Brackish 24 ppt	Eutrophic	<0.400	0.400	0.400	0.400	0.400	0.400
Sea Water 35 ppt	Oligotrophic	0.800	0.800	1.200	1.200	2.000	2.000
Sea Water 35 ppt	Eutrophic	0.800	0.800	1.200	1.200	1.200	1.200

TABLE 13

FIFTY PERCENT EFFECTIVE CONCENTRATIONS FOR MMH (as µℓ/ℓ) UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Water Quality		Day 6		Day 8		Day 10	
Salinity	Eutrophication level	Number	Volume	Number	Volume	Number	Volume
Fresh	Oligotrophic	0.360	0.500	0.390	0.550	0.410	0.610
Fresh	Eutrophic	0.270	0.350	0.320	0.440	0.380	0.530
Fresh	Very Eutrophic	0.300	0.300	0.430	0.480	0.430	0.430
Brackish 16 ppt	Oligotrophic	0.070	0.070	0.080	0.080		
Brackish 16 ppt	Eutrophic	0.095	0.097	0.115	0.117	0.145	0.153
Brackish 24 ppt	Oligotrophic	0.095	0.090	1.600	1.400	1.700	1.800
Brackish 24 ppt	Eutrophic	0.500	0.700	0.700	0.800	0.700	0.700
Sea Water 35 ppt	Oligotrophic	1.200	1.100	2.100	1.800	2.300	2.300
Sea Water 35 ppt	Eutrophic	1.200	1.200	1.500	1.400	1.600	1.600

TABLE 14

SAFE CONCENTRATION DOSES FOR UDMH (as μℓ/ℓ) UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Water Quality		Day 6		Day 8		Day 10	
Salinity	Eutrophication level	Number	Volume	Number	Volume	Number	Volume
Fresh	Oligotrophic	<0.80	2.00	2.00	2.00	2.00	2.00
Fresh	Eutrophic	0.50	5.00	3.01	5.00	0.50	3.00
Fresh	Very Eutrophic	0.50	0.50	0.50	5.00	0.50	1.00
Brackish 16 ppt	Oligotrophic	0.76	0.76	0.76	0.76	<0.76	0.76
Brackish 16 ppt	Eutrophic	<0.73	<0.73	0.73	0.73	0.73	0.73
Brackish 24 ppt	Oligotrophic	1.20	1.20	<1.20	1.60	<1.20	1.20
Brackish 24 ppt	Eutrophic			1.20	1.20	1.20	1.20
Sea Water 35 ppt	Oligotrophic	0.10	0.10	<0.05	<0.50	0.30	0.50
Sea Water 35 ppt	Eutrophic		0.10		0.30		0.10

TABLE 15

FIFTY PERCENT EFFECTIVE CONCENTRATIONS FOR UDMH (as µℓ/ℓ) UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Water Quality		Day 6		Day 8		Day 10	
Salinity	Eutrophication level	Number	Volume	Number	Volume	Number	Volume
Fresh	Oligotrophic	5.00	5.00	5.00	5.00	5.00	5.00
Fresh	Eutrophic	4.70	5.40	6.60	11.70	10.50	14.00
Fresh	Very Eutrophic	5.30	6.10	8.00	8.20	8.00	8.60
Brackish 16 ppt	Oligotrophic	1.10	1.00	0.90	1.20	1.10	1.60
Brackish 16 ppt	Eutrophic	0.90	0.80	1.00	1.00	1.30	1.20
Brackish 24 ppt	Oligotrophic	1.85	2.10	2.10	2.60	2.25	3.10
Brackish 24 ppt	Eutrophic			1.65	1.70	1.65	1.70
Sea Water 35 ppt	Oligotrophic	2.30	2.30	2.30	2.30	2.30	2.30
Sea Water 35 ppt	Eutrophic		0.99		1.02		1.01

TABLE 16

TOXICITY OF HYDRAZINE COMPOUNDS TO ALGAE

Type of Water Test Organism	Compound					
	Hydrazine		MMH		UDMH	
	SC	EC_{50}	SC	EC_{50}	SC	EC_{50}
Oligotrophic freshwater *S. capricornutum*	0.001	0.030	0.2	0.5	2.0	5.0
Oligotrophic seawater *D. tertiolecta*	0.0005	0.0008	0.8	1.1	0.1	2.3

APPENDIX: SUPPLEMENTAL INVESTIGATIONS

During the course of our work on the effects of hydrazine and hydrazine derivatives on unicellular algae, various technical problems became apparent. The appendix treats these various technical investigations which have been undertaken during the current year.

ANALYTICAL PROCEDURES

Analytical Procedures for MMH

Problems have been encountered with the analytical procedure for MMH determination in marine growth media. Specifically, the slope of the standard curve tended to change with increasing MMH concentration and the "desired" and "analyzed" concentrations were not in good agreement. The following factors were determined to be of critical importance in getting good chemical results:

1. Time and stability of the color complex. It was found that the MMH Dimethylaminobenzaldehyde color complex was not stable in a saline matrix but would develop to a maximum intensity and immediately start to fade. The development time which resulted in the best absorbance was found to be 55-60 minutes. Length of time between dosing the algal growth flasks and taking the sample for analysis was also more critical for MMH than for the other hydrazine compounds under similar conditions.
2. Salinity of the solvent. It was determined that the slope of the standard curve changed significantly when standards were prepared in deionized water or Artificial Sea Water. Absorbance values for a given MMH concentration were 25% to 30% higher in the salt water medium than in the deionized water.

In some of the first MMH analyses in ASW, standards were prepared in deionized water. When the initial "analyzed" concentrations are recalculated on the basis of the difference between the absorbance in deionized water and ASW, the concentrations are much closer to the "desired".

Standardization of Hydrazine Derivatives with their Sulfates

In 1976, four hydrazine compounds were sent to a laboratory in the area for gas chromatography/mass spectroscopy (GC/MS) analysis. Results indicated that all of the compounds had decomposed somewhat during storage. Since this type of analysis is expensive it was suggested by Dr. London and Major McNaughton that a hydrazine sulfate compound be used as the primary standard. This procedure was used to standardize hydrazine against hydrazine sulfate. Lot and batch numbers for compounds used were:

Hydrazine Sulfate

Min. 99.0% pure (LOT VCB, 5220, Mallinckrodt Chemical)

Hydrazine

(95+%) pure (LOT No. A3E, 902, Eastman Kodak Company)

Hydrazine Sulfate Working Standard

Conc. µℓ/ℓ	Absorbance
0	0
0.01	0.010
0.02	0.024
0.03	0.038
0.04	0.052
0.05	0.072
0.06	0.080
0.07	0.098
0.08	0.115
0.10	0.143

Hydrazine-Theoretical Concentrations and Absorbances

Theoretical Conc. µℓ/ℓ	Absorbance
0	0
0.03	0.038
0.05	0.068
0.08	0.110
0.10	0.132

Results

Hydrazine Conc. Theoretical µℓ/ℓ	Hydrazine Conc. Actual µℓ/ℓ	Purity %
0	0	
0.03	0.0287	95.71
0.05	0.0493	97.61
0.08	0.0780	96.52
0.10	0.0931	92.17
		$\bar{X}$ = 95.25%
		σ = ±2.374

Purity of hydrazine (LOT No. A3E, 902) is 95.25% with σ = ± 2.374. Hydrazine sulfate was assumed to be 99.0% pure.

This bottle of hydrazine was newly opened and the results indicate that the concentration is what the label states. The bottle of hydrazine from which the sample for GC/MS was taken was a different lot number and had been opened for some time. This lot number will also be standardized against the hydrazine sulfate.

PRELIMINARY STUDIES WITH AXENIC BATCH CULTURES

A new axenic culture of Selenastrum capricornutum was received and transferred into autoclave sterilized 100% SAAM medium. An attempt was made to purify the lab culture of Selenastrum used in the bacterized bioassays.

Three methods were used for purifying the Selenastrum culture:

1. Washing by centrifugation.

   A sterile centrifuge tube is filled with about 15 mℓ of an actively growing culture. This is spun for 45 to 60 seconds at 3000 rpm. The supernatant is discarded and the algae are resuspended in sterile SAAM medium. This procedure is repeated 10 times. Then the algal cells are streaked onto agar plates and colonies that show no sign of bacterial contamination are transferred to sterile SAAM medium. After algal growth is obtained in the liquid medium, the cultures will be tested for purity.

2. Antibiotic Formula I.

   A stock solution was prepared to contain the following:

   611 mg Pencillin "G"
   1000 mg Streptomycin Sulfate
   200 m Distilled water

   This solution was filter (0.22 µm) sterilized and 1 mℓ of the stock solution added for each mℓ of SAAM medium. Algae were transferred to the medium and left in contact with the antibiotics for 48 hours. At this time the algal cells were transferred to fresh sterile medium without antibiotics.

3. Antibiotic Formula II (Provasoli, 1958).

   A stock solution was prepared so that one mℓ contained the following:

   12,000 unit K Pencillin
   50 µg Chloramphenicol
   50 µg Polymyxin B
   60 µg Neomycin

   This solution was filter sterilized and 1.5 mℓ of stock added for each 100 mℓ of 100% SAAM medium seeded with Selenastrum capricornutum. Algae were left in contact with this antibiotic solution for seven days and then transferred to fresh sterile medium without antibiotics.

Of these three techniques, the washing procedure proved most reliable in providing in axenic culture which grew well.

Bioassays were set up to determine the reason for the difference in growth between the axenic culture of Selenastrum purchased from the Culture Collection of Algae and the bacterized Selenastrum used for the bioassays. Autoclave sterilized SAAM does not have as high algal growth potential as Millipore filtered SAAM but the purchased axenic culture did not grow nearly as well as the rebacterized culture (only 1-6% as much growth) in the autoclave sterilized SAAM. Because of this, the bioassays were conducted in Millipore filtered SAAM (33% and 100% nutrients) and utilized two types of flask closures:

1. Type of flask closure
2. Method of medium sterilization
3. Presence of bacteria which may provide vitamins

The most probable reason for the difference in growth is that the purchased culture is a different species or subspecies from the Selenastrum capricornutum used for the bacterized bioassays. Because of this difference in growth, the axenic culture obtained by cell washing will be used for the axenic algal bioassays.

Good algal growth of both axenic (by washing techniques) and bacterized cultures was obtained in autoclave sterilized 100% Standard Algal Assay Medium (SAAM) using the following procedure. Growth medium is prepared with all compounds except phosphate (K_2HPO_4) and trace metal solution. The incomplete medium is dispensed into the bioassay flasks and autoclave sterilized at 121°C, 15 psi for 20 minutes. Phosphate and trace metal solutions are autoclave sterilized separately and added to the incomplete medium after it has cooled. Since bacterized cultures of Selenastrum achieve normal maximum standing crop when the medium is prepared in this way, this method of preparation will be used for the axenic bioassays.

REFERENCES

American Public Health Association, 1975. Standard Methods for the Examination of Water and Wastewater (14th Edition). American Public Health Association, Washington, D.C.

Droop, M.R., 1969. Methods in Microbiology. Academic Press, New York.

Pinkerton, Mildred K., et al., December 1961. A Colorimetric Determination for 1, 1-Dimethylhydrazine in Air, Blood, and Water ASD-TR-61-708 (AD273986). Aeronautical Systems Division, Wright-Patterson Air Force Base, Ohio.

Provasoli, L., 1958. Effect of Plant Hormones on Ulva, Biological Bulletin, 114, 375-384.

Reynolds, B.A. and A.A. Thomas, April 1964. Determination of Hydrazine and 1-Methylhydrazine in Blood Serum AMRL-TDR-62-24 (AD285321L). Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio.

Scherfig, J., et al., November 1977. Use of Unicellular Algae for Evaluation of Potential Aquatic Contaminants. Second Annual Report AMRL-TR-77-53.

Stein, Janet R., 1973. Handbook of Phycological Methods. Cambridge University Press, New York.

United States Environmental Protection Agency, 1971. Algal Assay Procedure: Bottle Test. Pacific Northwest Environmental Research Laboratory, Corvallis, Oregon.

United States Environmental Protection Agency, December 1974. Marine Algal Assay Procedure: Bottle Test. Pacific Northwest Environmental Research Laboratory, Corvallis, Oregon.

☆U.S.Government Printing Office: 1979 — 657-002/329